SUBTLE MITOCHONDRIAL MUTATIONS AS TUMOR MARKERS

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TECHNICAL FIELD OF THE INVENTION

The invention is related to the area of cancer genetics. In particular it is related to somatic mutations associated with cancer.

BACKGROUND OF THE INVENTION

The human mitochondrial genome is a 16 kilobase circular, double stranded DNA that encodes 13 polypeptides of the mitochondrial respiratory chain, 22 transfer RNAs, and two ribosomal RNAs required for protein synthesis. The mitochondrial genome is particularly susceptible to mutations because of the high level of reactive oxygen species generated in the organelle coupled with a low level of DNA repair (7-10). Surprisingly, there has been little detailed analysis of alterations in human mitochondrial DNA in cancer, although there are indications that mitochondria may be involved in neoplasia and apoptosis (2-6) as well as cancerous growth (1). Thus, there remains a need in the art to identify a correlation of alterations in human mitochondrial DNA with cancer.

SUMMARY OF THE INVENTION

It is an object of the invention to provide a method to aid in detecting the presence of tumor cells in a patient. This and other objects of the invention are provided by one or more of the embodiments described below.

One embodiment of the invention provides a method of screening patients for the presence of tumor cells. The method comprises a step for determining the presence of a single basepair mutation in a mitochondrial genome of a cell sample of a patient. If a single basepair substitution is found in a cell sample of the patient which is not in normal tissue of the patient, the patient is identified as having a tumor.

The invention thus provides the art with new methods of detecting and tracing tumors by examining mitochondrial DNA for the appearance of somatic mutations.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows examples of mitochondrial DNA mutations. The sequence of the mitochondrial genome was determined in normal cells, primary tumors, and tumor cell lines from the same patients. Arrows indicate the G-to-A transition (antisense strand) at codon 121 of the COX subunit I gene in line V425, an A insertion within the (A)8 tract of the ND5 gene in line V425, a T-to-C transition at codon 1 of the ND1 gene in line 478, and a G-to-A transition (antisense strand) at codon 142 of the COX subunit II gene in line V429.

Figure 2A to Figure 2C depict somatic cell fusions. Fig. 2A. Confirmation of successful nuclear fusion using nuclear genomic DNA polymorphisms from the indicated lines. Fig. 2B. Analysis of the mitochondrial DNA utilizing the T-to-C variant at nucleotide 4,216 that creates a recognition site for Nla III (CATG). The C variant, giving rise to 376 and 231 fragments following restriction digest of a 1,140 bp PCR product, is present only in DLD-1 cells. Fig. 2C. Time course over which replicative advantage of DLD-1 mitochondria is evident. Initially, HCT116 cell mitochondria were slightly over represented in the fusions, but a shift towards DLD-1 mitochondria was evident within five days and this process was

complete between 15 and 60 days. DNA was isolated and the mitochondria were analyzed by Nla III digestion on the indicated days after cell fusion.

DETAILED DESCRIPTION OF THE INVENTION

The inventors have found that the presence of subtle mutations in the mitochondrial genome can be used as a means to trace the presence, spread, metastasis, growth, or recurrence of a tumor in a patient. Such subtle mutations include single basepair substitutions, single basepair insertions, and single basepair deletions. Single basepair substitutions can be either transitions or transversions, although the former are more frequent. Detection of such mutations can be useful to screen for the initial appearance of a tumor as well as the recurrence of a previously identified tumor. The methods are particularly suited to monitor anti-cancer therapy, recurrence, metastasis, and completeness of surgical removals.

A single basepair substitution is the substitution of a single nucleotide base with a different nucleotide base at the same position, with the corresponding substitution of the complementary base on the other strand of the DNA. While any single basepair substitution is conceivable within the scope of the invention, the most frequently encountered substitutions are those which are consistent with oxidative damage, such as T to C or G to A transitions. The mutations can appear in protein coding regions or in regions which encode ribosomal or transfer RNAs.

The homoplasmic property of most mutant mitochondrial genomes from tumors permits the ready detection of such mutations within a sample of mitochondrial DNA from a patient. Homoplasmic mutations are those which appear in essentially all of the copies of the mitochondrial genome within a given cell or tissue. However, heteroplasmic mutations, which are those appearing in only a fraction of the mitochondrial genomes of a cell or tissue, are also suitable for use with the invention.

Any cell sample can be tested from a patient who has cancer or is suspected of having cancer. Suitable cell samples include, but are not limited to, tissue from a growth suspected or known to be cancerous, tissue adjacent to a resection of a tumor, and tissue distant from the site of a tumor, such as lymph nodes which are

suspected of bearing metastatic cells. Cells can also be obtained from bodily fluids or secretions, *e.g.*, blood, urine, sputum, saliva, or feces, which may contain cancerous cells or metastatic cells. Cell samples can also be collected from other bodily secretions and tissues as is known in the art. A cell sample can be collected from suspected or known cancerous tissue or from bodily fluids or secretions harboring cancer cells as well as from suspected or known normal tissue or bodily fluids or secretions harboring normal cells.

In order to detect mutations of the mitochondrial genome from a cell sample of a patient, mitochondrial DNA can be isolated from the cell sample using any method known in the art. One way of identifying subtle mutations involves sequencing the mitochondrial DNA. This can be done according to any method known in the art. For example, isolated mitochondrial DNA can be cleaved using endonucleases into overlapping fragments of appropriate size for sequencing, *e.g.*, about 1-3 kilobases in length, followed by polymerase chain reaction (PCR) amplification and sequencing of the fragments. Examples of DNA sequencing methods are found in Brumley, R.L. Jr., and Smith, L.M., 1991, Rapid DNA sequencing by horizontal ultrathin gel electrophoresis, *Nucleic Acids Res.* 19:4121-4126 and Luckey, J.A., Drossman, H., Kostihka, T.; and Smith, L.M., 1993, High-speed DNA sequencing by capillary gel electrophoresis, *Methods Enzymol.* 218:154-172. Amplification methods such as PCR can be applied to samples as small as a single cell and still yield sufficient DNA for complete sequence analysis. The combined use of PCR and sequencing of mitochondrial DNA is described in Hopgood, R., Sullivan, K.M., and Gill, P., 1992, Strategies for automated sequencing of human mitochondrial DNA directly from PCR products, *Biotechniques* 13:82-92 and Tanaka, M., Hayakawa, M., and Ozawa, T., 1996, Automated sequencing of mitochondrial DNA, *Methods Enzymol.* 264:407-21.

Mutations can first be identified by comparison to sequences present in public databases for human mitochondrial DNA, *e.g.*, at http://www.gen.emory.edu/mitomap.html. Any single basepair substitution identified in the sample DNA compared to a normal sequence from a database can

be confirmed as being a somatic mutation as opposed to a polymorphic variant by comparing the sample mitochondrial DNA or sequences obtained from it to control cell mitochondrial DNA from the same individual or sequences obtained from it. Control cells are isolated from other apparently normal tissues, *i.e.*, tissues which are phenotypically normal and devoid of any visible, histological, or immunological characteristics of cancer tissue. A difference between the sample and the control identifies a somatic mutation which is associated with the tumor.

An alternative to serially sequencing the entire mitochondrial genome in order to identify a single basepair substitution is to use hybridization of the mitochondrial DNA to an array of oligonucleotides. Hybridization techniques are available in the art which can rapidly identify mutations by comparing the hybridization of the sample to matched and mismatched sequences which are based on the human mitochondrial genome. Such an array can be as simple as two oligonucleotide probes, one of whose sequence matches the wild-type or mutant region containing the single base substitution (matched probe) and another whose sequence includes a single mismatched base (mismatch control probe). If the sample DNA hybridizes to the matched probe but not the mismatched probe, it is identified as having the same sequence as the matched probe. Larger arrays containing thousands of such matched/mismatched pairs of probes on a glass slide or microchip ("microarrays" or "gene chips") are available which are capable of sequencing the entire mitochondrial genome very quickly. Such arrays are commercially available. Review articles describing the use of microarrays in genome and DNA sequence analysis and links to their commercial suppliers are available at www.gene-chips.com.

The invention can be used to screen patients suspected of having cancer for the presence of tumor cells. A cell sample is first obtained from a suspected tumor of the patient, or is obtained from another source such as blood or lymph tissue, for example, if metastasis is suspected. The cell sample is tested to determine the presence of a single basepair mutation in mitochondrial DNA from the cell sample using the techniques outlined above. Optionally, a cell sample from normal, non-cancerous cells or tissue of the patient is also obtained and is tested for the

presence or absence of a single basepair mutation in mitochondrial DNA. If a single basepair mutation is determined which is not present in a cell sample from normal tissue of the patient, then the mutation is a somatic mutation and the presence of tumor cells in the patient is indicated. If one or more single basepair mutations are determined in the mitochondrial genome of the cell sample of the patient, then the patient is identified as having a tumor. As in any diagnostic technique for cancer, to confirm or extend the diagnosis, further diagnostic techniques may be warranted. For example, conventional histological examination of a biopsy specimen can be performed to detect the presence of tumor cells, or analysis of a tumor-specific antigen in a blood or tissue sample can be performed.

The method outlined above can be practiced either in the situation where the somatic mutation is previously known or previously unknown. Based on the inventors' findings, the identification of a previously unknown somatic mutation in a mitochondrial genome of a cell of a patient is likely to indicate the presence of tumor cells in the patient. Therefore, the method can be practiced even in the absence of prior knowledge about any particular somatic mutation. The method can also be carried out subsequent to the discovery of a somatic mutation in a mitochondrial genome of a cell of the patient or of another patient. In this case, a previous association of the somatic mutation with the presence of a tumor in the patient or in another patient strongly indicates the presence of tumor cells in the patient. It may also indicate the recurrence of a tumor or the incomplete prior removal of cancerous tissue from the patient.

The effectiveness of therapy can be evaluated when a tumor has already been identified and found to contain a single basepair substitution in the mitochondrial genome. Once a single basepair mutation has been identified in the mitochondrial DNA of a tumor of the patient, further tumor cells can be detected in tissue surrounding a resection or at other sites, if metastasis has occurred. Using the methods outlined above, the recurrence of the tumor or its incomplete removal can be assessed. Similarly, if a tumor has been treated using a non-surgical method such as chemotherapy or radiation, then the success of the therapy can be evaluated at later times by repeating the analysis. The step for

determining the presence of a single basepair mutation in a mitochondrial genome of a cell sample of a patient can be performed 1, 2, 3, 4, 5, 6, 8, 10, or more times in order to monitor the development or regression of a tumor or to monitor the progress or lack of progress of therapy undertaken to eliminate the tumor.

Upon repeated analyses, the step for determining the presence of a single basepair mutation is simplified, because only a well defined and limited region of the genome need be sequenced. Using the hybridization method, for example, it is possible to evaluate the presence of the mutation with only a single matched/mismatched pair of oligonucleotide probes in the array. In the event that a mixture of genotypes is observed, it is possible to obtain quantitative information about the relative amount of each mitochondrial genotype using techniques known to the art, *e.g.*, hybridization. Quantitative analysis can reveal changes in the relative proportion of tumor to normal cells in a tissue over time or in response to therapy.

The methods described above were used to study somatic mutations in mitochondrial DNA from human colorectal tumor cells (see Examples 1 and 2). The mutations observed generally were transitions, affecting G residues, which are the preferred targets for oxidative damage to DNA in general and mitochondrial DNA in particular (at least *in vitro*) (12, 13, 17, 18). This mutational spectrum supports the idea that the mitochondrial DNA mutations resulted from the reactive oxygen species continually generated in mitochondria. Sequence analyses of nuclear genes from the same ten cell lines studied for mitochondrial DNA mutations indicated that the prevalence of mutations is at least ten-fold higher in the mitochondrial genome than in the nuclear genome of those cells. Previous experiments have demonstrated large deletions in the mitochondrial DNA of some tumors (19-23), rather than the subtle mutations observed here. No deletions were observed in the cell lines studied here, despite several attempts to find them using multiple primer pairs in PCR-based strategies. The literature reveals no previous attempts to search for subtle mutations of mitochondrial genomes by complete sequencing.

The mutations reported in Table 1 were mostly homoplasmic, while the

deletions previously observed in tumor cells or normal cells of aging individuals were generally heteroplasmic, present only in a small proportion of the mitochondrial population (19-24). The results presented here are not at variance with a previous study in which no somatic mutations in 200 bp of D-loop sequence were found. This D loop sequence contains promotor elements for transcription of the mitochondrial genome, while the mutations discovered by the inventors were confined to regions encoding mitochondrial proteins or rRNA.

The striking and unexpected homoplasmy of the mutations identified by the inventors indicates significant selection at several levels. First, the somatically mutant mitochondrial genome must be replicated better than that present in the germ-line. Previous experiments have indicated that replication of mitochondria can be controlled individually, in that signals from aberrantly functioning mitochondria induce their overreplication, perhaps in a compensating effort (25). The fusion experiments of Example 2 demonstrate that the process of mitochondrial selection in tumor cells can take place rapidly (Fig. 2C). Over the thousands of generations required for tumorigenesis *in vivo*, this process could easily result in the replacement of all mitochondrial genomes within the cell with a mutant form. This cell could then overtake the population through clonal growth, either because the aberrant mitochondria themselves endowed the cell with a selective growth advantage or because that cell sustained a nuclear gene mutation providing such an advantage.

This explanation invokes the idea that the mitochondrial mutations may themselves have a functional effect. It is unlikely that most of the observed somatic mutations result in major perturbations of mitochondrial function, as oxygen consumption and the respiratory chain enzymatic activities of several of the lines listed in Table 1 were largely normal. Instead, these mutations, perhaps in concert with polymorphic variations in mitochondrial DNA, probably result in subtle changes which might generate slightly higher levels of ROS. It has been shown that low levels of ROS are highly mitogenic, while high ROS levels are toxic (9). Regardless of the mechanism for their selection, however, the mutations that have been identified represent a previously unrecognized alteration in tumor

cells that could have significant effects on the cellular processes controlled by mitochondria. Their homoplasmy raises fascinating questions about the control of mitochondrial DNA at the intramitochondrial, intracellular, and cellular population levels. It indicatea that a single cell with a mutant mitochondrial genome had acquired a selective growth advantage during tumor evolution, allowing it to become the predominant cell type within the tumor cell population. Furthermore, it is important to appreciate that cells, including the colorectal cancer cell lines used here, each contain hundreds of mitochondria, and each mitochondrion contains one to ten DNA molecules (14). The homoplasmy therefore additionally indicates that each mutant mitochondrial genome had a replicative advantage within the particular mitochondrion in which it occurred, and that this mitochondrion had selectively proliferated over other mitochondria in the same cell. Alterations of tumor mitochondrial DNA may also provide clues to their environmental or genetic background, a hypothesis that can be tested in the future using DNA chip technologies (26).

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific examples, which are provided herein for purposes of illustration only and are not intended to limit the scope of the invention.

EXAMPLE 1

Identification of somatic mutations in mitochondrial DNA of human colorectal cancer cells.

To determine whether mutations of the mitochondrial DNA were present in human colorectal tumors, the entire mitochondrial genome was PCR-amplified from ten human colorectal cancer cell lines in 1-3 kb overlapping fragments and the PCR products completely sequenced. The use of large PCR products excluded the possibility that nuclear pseudogenes would complicate this analysis (11).

Cell lines and tumors. Derivation and maintenance of the VACO lines has been previously described (27). The DLD-1, HCT116, SW837 and HT29 human

colorectal cancer cell lines were obtained from ATCC and maintained in McCoy's medium (Gibco, BRL) supplemented with 10% fetal bovine serum (Hyclone) and antibiotics (Gibco, BRL).

DNA purification, PCR amplification and sequencing. Cellular DNA from cell lines, from primary tumors, and from normal colonic mucosa was isolated as previously described (28). Overlapping fragments (1-3 kb each) of the mitochondrial genome were amplified by PCR using this DNA as template. The sequencing method allowed the detection of any mutation present in >25% of the mitochondrial DNA molecules within a given sample. In selected cases, the validity of the sequence data was confirmed using purified mitochondrial DNA as templates. To confirm the mutations in the primary tumors, smaller PCR fragments were generated from the DNA purified from microdissected, paraffin-embedded samples. Manual sequencing of the DNA fragments was performed using Thermosequenase (Amersham) and a Genomyx electrophoresis apparatus (Beckman).

Sequence analysis. The sequences obtained were first compared to those recorded in the mitochondrial databank at www.gen.emory.edu/mitomap.html. Eighty-eight sequence variants were identified (4 - 31 per tumor) that were not recorded in this databank. These included 27 variants which were predicted to alter the amino acid sequence of the encoded protein, 48 variants which were in protein coding regions but predicted to be silent, and 13 which affected rRNA or tRNA genes.

The database search provided only preliminary evidence for mutations, however, as it could not distinguish somatic mutations from rare germ-line variants. To make this distinction, mitochondrial DNA sequences were determined from normal colons from the same patient. This analysis showed that at least seven of the lines contained true somatic mutations. Three of the lines contained a single mutation, while four others contained two or three mutations (Table 1).

Each of the 27 sequence variants predicted to result in amino acid changes was evaluated to determine its somatic nature; of these, eight were found to be

somatic and 19 were found in the germline of the same patient. Of the 13 variants in rRNA or tRNA genes, nine were evaluated in this way and four were found to be somatic. Twenty-five of the 48 silent mutations were also evaluated, and none of these were found to be somatic.

Of the 12 somatic mutations identified, eight were in protein encoding genes and four were in rRNA genes (Table 1). Eleven were nucleotide substitutions and one was a single bp insertion. Of the eight mutations in protein-encoding genes, one was a nonsense mutation, one was a 1-bp insertion, and six were missense mutations (Table 1). All but one of the 11 nt substitutions were T to C or G to A transitions. This mutational spectrum is fully consistent with the known mutagenic spectra of oxidative damage (12, 13).

To determine whether these mutations arose *in vivo* rather than during the process of cell culture, DNA was purified from five of the primary tumors from which the lines were derived (in two cases, no primary tumors were available). In every evaluable case, the mutation was found in the primary tumor as well as in the cell line (examples in Fig. 1).

Surprisingly, each of the 12 mutations was present in a major portion of the mitochondrial DNA molecules, and in ten of the 12 cases, the mutations were homoplasmic, *i.e.*, apparently present in every mitochondrial genome (Table 1). This homoplasmy was observed both in the primary tumors as well as in the cell lines (Fig. 1).

EXAMPLE 2

Proliferation of mitochondria harboring somatic mutations.

Cell fusion experiments have indicated that mitochondria from tumor cells can selectively proliferate when such cells are fused to normal cells (15). The inventors sought to determine whether a similar mitochondrial dominance could be observed upon fusion between two colorectal cancer cell lines. Attempts to fuse the lines studied for mitochondrial mutations were unsuccessful for technical reasons. Therefore, more commonly used colorectal cancer cell lines were employed in which intercellular fusions are possible (16).

Geneticin-resistant DLD-1 cells were fused to hygromycin-resistant subclones of each of three different colorectal cancer cell lines (HCT116, HT29, and SW837). Geneticin- or hygromycin-resistant clones were derived through transfection of appropriate plasmid vectors. Approximately 10^6 hygromycin-resistant cells were mixed with an equal number of neomycin-resistant cells and fused by PEG treatment as described (16). Hybrids were selected in standard growth medium containing 1 mg/ml geneticin and 0.25 mg/ml hygromycin (DLD-1-HCT116 fusion), 1.5 mg/ml geneticin and 0.6 mg/ml hygromycin (DLD-HT29 fusion) and 1 mg/ml geneticin and 0.25 mg/ml hygromycin (DLD-SW837 fusion). Successful fusions were verified by nuclear genotyping. Allelotyping was carried out as described (29) using the primer pair wg1g5A/wg1g5B or MapPair primers for D19S591 and D16S764 (Research Genetics). Amplified fragments were resolved by electrophoresis in 8% polyacrylamide gels. Reactions using radioactively labeled primers were separated on a 4.5 % sequencing gel (Genomyx), while reactions using fluorescence labeled primers were analyzed on an ABI Sequencing System (Perkin-Elmer).

The success of fusion was demonstrated using nuclear genomic polymorphisms (Fig. 2A). Complete sequencing of the mitochondrial genomes revealed 3 - 7 potential variants in each line; it could not be determined which of these were somatic, as normal cells derived from the individuals from whom the cell lines were derived were not available. However, these variants provided a convenient method to trace the fate of the mitochondrial DNA in the fusions. In particular, a T to C variant at nucleotide 4,216 was used that creates a restriction endonuclease recognition site for Nla III (Fig 2B). The C variant was present in DLD-1 cells but not in any of the other three lines. As shown in Fig. 2B, the DLD-1 mitochondria were "dominant" over the other mitochondria in each of the fusions. All three tested clones derived from DLD-1/HCT116 fusions contained mitochondria exclusively of DLD-1 origin. A pool of over 100 stable clones from this fusion also contained only mitochondria from DLD-1cells. DLD-1 mitochondria were also dominant to those from HT29 and SW837 cells,

contributing either all or the majority of mitochondrial genomes in the clones tested (Fig. 2B).

To determine the time course over which the replicative advantage of DLDl-1 mitochondria occurred, pooled clones from DLD-1/HCT116 fusions were followed. At the initiation of this experiment, there was a mixture of mitochondrial genomes, with a slight excess of the mitochondria from HCT116 cells. Within five days, a skewing towards DLD-1 mitochondria was evident, and a major shift occurred between 15 and 60 days after fusion, by which time only DLD-1 mitochondria remained in the hybrids (Fig 2C). Whether it was strictly the mitochondria, or a combination of nuclear and mitochondrial factors, that was responsible for the selection of DLD-1 mitochondria could not be determined. However, these experiments clearly documented that tumor mitochondria of one type can have a significant replicative advantage over other types, and are consistent with other experiments documenting the potential for mitochondrial dominance (15).

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Table 1 Summary of mtDNA mutations

Tumor*	Position	DNA	Protein	Gene
V478	710	T→C	-	12S rRNA
"	1738	T→C	-	16S rRNA
"	3308	T→C	M1T	ND1
V429	8009	G→A	V142M	COX subunit II
"	14985	G→A	R80H	CYT b
"	15572	T→C	F276L	CYT b
V441	9949	G→A	V2481	COX subunit III
V456	10563	T→C	C32R	ND4L
V425	6264	G→A	G121trun	COX subunit I
"	12418	insA	K28frameshift	ND5
V451	1967	T→C	-	16S rRNA
V410	2299	T→A	-	16S rRNA

*All the mutations were homoplasmic except V451 T11967C and V410 T2299A, which were present in ~50% of the mitochondrial DNA molecules.